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Research report

# Evaluating the validity of blood-based membrane potential changes for the identification of bipolar disorder I

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**Abstract**

*Objective:* The objective of this study was to develop a diagnostic blood test for bipolar disorder I using membrane potentials as biological markers.
*Methods:* We measured the fluorescence intensity of a dye sensitive to membrane potential in whole blood samples from bipolar I, unipolar, schizophrenic patients, and psychiatrically normal controls. Patients were diagnosed through structured clinical interviews according to DSM-IV. Both the *t*-test and logistic regression analysis were used to analyze the data.
*Results:* The membrane potential as indicated by the fluorescence intensity of the membrane potential dye in blood cells drawn from patients with bipolar disorder I was significantly different from the blood cells drawn from unipolar and schizophrenic patients, and from psychiatrically normal controls ($P<0.001$). The specificity and sensitivity were determined to be 0.88 and 0.78 respectively which compared well with the state of the art diagnostic techniques for other diseases. Logistic regression analysis revealed that the membrane potential was a reliable predictor which could be used as a diagnostic marker for bipolar I.
*Conclusions:* These results indicate that the membrane potential of blood cells can be used as a diagnostic marker to augment the DSM-IV diagnosis of bipolar disorder I. Expanded clinical trials are needed to establish this technique for general use.


*Keywords:* Bipolar disorder I; Membrane potential; Whole blood cells; Diagnostic tests; Sensitivity; Specificity

## 1. Introduction

The diagnosis of bipolar disorder can be difficult and many patients can go undiagnosed or misdiagnosed for a decade or more. There is a need for a clinical blood test to augment the DSM IV diagnosis (Lish et al., 1994; Suppes et al., 2001; Hirschfeld et al., 2003; Ghaemi et al., 2000) among others and misdiagnosis of bipolar illness can have adverse consequences on the course of the illness. Given the wealth of literature suggesting a biologic and/or genetic abnormality in this disorder, a biological diagnostic test would be a reasonable expectation.

Sodium and potassium-activated adenosine triphosphatase, ($Na^+$, $K^+$-ATPase or sodium pump), whose activity is essential to maintain the homeostasis of membrane potential, has been investigated for its possible involvement in the pathophysiology of bipolar disorder I (el Mallakh and Wyatt, 1995). However, this has been an unsettled and controversial subject in the field for many years. $Na^+$, $K^+$-ATPase activity has been

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variously reported to be increased, decreased, or unchanged in bipolar patients. In 1997, Looney et al. conducted a meta-analysis of the available literature on erythrocyte $Na^+$, $K^+$-ATPase activity in bipolar disorder patients and concluded that it is lower in bipolar patients (Looney and el Mallakh, 1997). The question of exactly how the $Na^+$, $K^+$-ATPase plays a role in bipolar disorder remains unanswered. El-Mallakh et al. measured the trans-membrane potential in leukocytes from hospitalized bipolar patients and observed that the trans-membrane potentials in lymphocytes of the bipolar patients were hyperpolarized compared with normal controls and euthymic patients on lithium (el Mallakh et al., 1996). However, Buss et al. measured the membrane potentials of cultured lymphoblasts and concluded that there was no significant difference in membrane potentials in lymphoblasts among bipolar patients, their siblings and normal controls (Buss et al., 1996). Recently Thiruvengadam analyzed the role of lithium in depolarizing the resting membrane potential of neurons using the Goldman–Hodgkin–Katz equation for membrane potential and showed that the addition of lithium would depolarize the membrane potential (Thiruvengadam, 2001, 2004).

In addition, a smaller but significant increase in $Na^+$, $K^+$-ATPase density (number of molecules/unit area) after incubation for 72 h in ethacrynate (a diuretic), or in lithium has been observed in blood cells drawn from bipolar I patients compared to blood cells from unaffected individuals (Wood et al., 1991). Cherry and Swann showed that the potassium uptake in cultured lymphoblasts from affected bipolar patients was much lower as compared to that of siblings as well as matched normal subjects (Cherry and Swann, 1994). Li and El-Mallakh found that ethacrynate-treated lymphoblastoid cells from bipolar individuals were unable to up-regulate $Na^+$, $K^+$-ATPase as do cells from psychiatrically normal controls (Li and el Mallakh, 2004). These results suggest that the changes in membrane potential, reflecting the changes in $Na^+$, $K^+$-ATPase regulation, may occur in blood cells of affected patients compared to those in unaffected control subjects.

Our preliminary results indicated that the membrane potentials of cultured lymphoblasts from bipolar patients were significantly different from that of controls and siblings. Such direct measurements of membrane potentials using fluorescent dyes are subject to several limitations as discussed later in this paper. This led us to develop a ratio method in which we measured the fluorescence intensity of a membrane potential dye in cultured lymphoblasts obtained from controls, siblings and bipolar patients in a reference (sometimes called regular) buffer, in a $K^+$-free buffer (reference buffer minus $K^+$), and in the $K^+$-free buffer with ethacrynate. The ratios of fluorescence intensity between cells incubated in $K^+$-free buffer over reference buffer and those of cells incubated in $K^+$-free buffer with ethacrynate over reference buffer were determined. These ratios were significantly different in bipolar patients as compared to those from controls and siblings. We also tested whether similar changes occur in blood cells of bipolar patients and evaluated whether these changes would have a diagnostic use.

## 2. Methods

### 2.1. Cell cultures

Immortalized lymphoblasts from bipolar patients, their siblings and controls were obtained from the Human Genetic Mutant Cell Repository (Coriell Institute for Medical Research, Camden NJ). Six samples of cells each from bipolar patients, their siblings and unrelated control subjects were used. These cells were grown at 37 °C in RPMI 1640 culture medium (GIBCO, Life Technologies, Gaithersburg, MD, USA) with 15% fetal bovine serum and 1× penicillin/streptomycin.

### 2.2. Membrane potential measurements

Cultured cells grown in suspension were centrifuged at 210×*g* for 5 min at room temperature and the cell pellets were suspended in 3 ml of reference buffer (5 mM KCl, 4 mM $NaHCO_3$, 5 mM HEPES, 134 mM NaCl, 2.3 mM $CaCl_2$, and 5 mM glucose). Cells were counted and the required number of cells was suspended in 3 ml of reference buffer. The cells were incubated with a voltage sensitive fluorescent dye [3, 3′-dihexyloxacarbocyanine iodide [DiOC6 (3) (Molecular Probes Inc. Eugene, OR, USA)] for 30 min at room temperature. The cell suspension was centrifuged and re-suspended in the same buffer without the dye. Membrane potential as indicated by the fluorescence intensity of the dye was measured using a fluorescence spectrometer (F2500 Hitachi, Japan). The intensity of fluorescence was measured at an excitation wavelength of 488 nm and emission at 530 nm. The time of recordings varied from 10 s to 1500 s depending upon the experiment.

The fluorescent dye, DiOC6(3), is used to measure plasma and mitochondrial membrane potential. However, the concentration of the dye that is used to measure mitochondrial membrane potential is < 1 nM (Rottenberg and Wu, 1998). In our experiments we used the dye at μM concentrations. It is documented that DiOC6(3) mostly reports plasma membrane potential changes at concentrations higher than 40 nM, whereas at concentrations lower than 1 nM it mostly reports mitochondrial membrane

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Table 1
Patient demographics for blind trials

| Ethnicity and gender | Total | Bipolar I | Unipolar | Schizophrenic | Control | Age (yrs) |
|---|---|---|---|---|---|---|
| White Male (WM) | 23 | 9 | 4 | 3 | 7 | 18–67 |
| White Female (WF) | 21 | 14 | 2 | 2 | 3 | 18–65 |
| Black Male (BM) | 6 | 0 | 0 | 4 | 2 | 30–60 |
| Black Female (BF) | 12 | 3 | 6 | 1 | 2 | 30–60 |
| Asian Male (AM) | 3 | 0 | 0 | 0 | 3 | 25–35 |
| Asian Female (AF) | 2 | 1 | 0 | 0 | 1 | 35–45 |
| Total | 67 | 27 | 12 | 10 | 18 | |

potential changes (Nicholls and Ward, 2000). Thus it appears that the positively charged dye at lower concentrations partitions more into mitochondria due to the higher negative potential of mitochondria whereas at higher concentrations the fluorescence intensity might represent the negative potentials of both mitochondria and plasma membrane. In addition, this dye has been used previously by other investigators to measure membrane potential of erythrocytes and lymphocytes including bipolar cells (Pratap et al., 1990; Waczulikova et al., 2000; el Mallakh et al., 1996).

We used a ratio method which eliminates distortions of data caused by photo bleaching and variations in probe loading and retention, as well as instrumental factors such as illumination stability and sensitivity of the detector. The ratio indicates the differences in the potentials among specific samples in two different buffers under identical conditions. Our approach is based on the following considerations:

The relationship between fluorescence intensity and membrane potential can be stated as

$$I = CV \tag{1}$$

where $I$ is the fluorescence intensity, $V$ is the membrane potential, and $C$ is the proportionality constant. The proportionality constant $C$ depends upon the dye loading, cell concentration, temperature and other experimental errors. By doing experiments in two different buffers (regular and $K^+$-free) simultaneously we cancel out $C$ as shown below:

$$I_1 = CV_1 \tag{2}$$

for the first measurement and

$$I_2 = CV_2 \tag{3}$$

for the second measurement.

If we take a ratio between Eqs. (3) and (2)we get

$$\frac{I_2}{I_1} = \frac{V_2}{V_1} \tag{4}$$

For ratio measurements, equal volumes of cell suspension were added to either the reference buffer or the $K^+$-free buffer or the $K^+$-free buffer with ethacrynate and incubated with the dye for 30 min. The intensity of fluorescence was then measured under identical conditions except for the buffer. We found that the treatment with ethacrynate would augment the difference in the ratio of fluorescence intensity of the membrane potential dye in bipolar subjects compared to other subjects. Therefore, the florescence intensity ratio in $K^+$-free buffer with ethacrynate (at pH = 6.8) over that in reference (regular) buffer (at pH = 7.4) was used in the clinical trials.

### 2.3. Clinical trial

The clinical trial was performed strictly following the regulations and guidelines of the Internal Review Board (IRB). Informed consent of each subject was obtained before the blood was drawn. The confidentiality of the patient records was ensured according to the IRB procedures. The investigator performing the membrane potential measurements knew only the sample number but not the patient history or the diagnosis. The study included blood samples from patients diagnosed as bipolar disorder I ($n$=27), major depression ($n$=12), schizophrenia ($n$=10), and psychiatrically normal controls ($n$=18). Table 1 shows the ethnicity and gender of these blind trial groups. A total of 67 samples including 32 males and 35 females were tested. 18 were normal controls with no known psychiatric illness and 49 were patients. Of the 49 patients, 30 were inpatients who were stabilized and on medication during blood draw. The remaining 19 were outpatients under the care of a clinical psychiatrist in the local community. The ages ranged from 21 to 70 years. Seventeen of the bipolar patients were on lithium while 9 were on valproate and one was on topiramate after electroconvulsive therapy. Two of the bipolar patients were diabetics. If a patient matched a diagnosis, structured clinical interviews according to DSM-IV guidelines were performed (Bowden, 2002).

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Whole blood samples were drawn from the subjects and were stored at 4 °C. All the samples were tested within 24 h. The membrane potentials were measured both in the $K^+$ free buffer with ethacrynate and in the reference buffer without ethacrynate. First fluorescent dye was added to both suspensions simultaneously and incubated for 30 min. Then the cell suspension loaded with the dye was centrifuged, drained and re-suspended in the respective buffers. The intensity of fluorescence was measured for 10 seconds and the ratio of the intensity in the $K^+$-free buffer with ethacrynate over the intensity in the reference (regular) buffer was calculated.

### 2.4. Statistical analysis

Based on the results obtained in the open trial, we determined the sample size to be 5 with a power of 0.9, an alpha of 0.05, difference in mean of 0.08, and a standard deviation of 0.03. However, we conservatively tested a total of 67 samples including 18 normal controls, 10 schizophrenics, 12 unipolars and 27 bipolar I (40 non-bipolars and 27 bipolars). The statistical significance of the differences between bipolar groups and non bipolar groups was determined by $t$-test (15). Logistic regression was used to assess the predictive power of the membrane potential ratios for the various diagnoses (16). A binomial logistic regression model can be used to calculate the probability that the "patient" is bipolar I or not. Binomial logistic regression is a form of regression which is used when the dependent variable is dichotomous and the independents are of any type (Tabachnick and Fidell, 2001). Logistic regression applies maximum likelihood estimation after transforming the dependent into a logit variable (the natural log of the odds of the dependent occurring or not). The logit for these data is given by:

$$\text{Logit } P = 997.042-(1223.223^{*}\text{BPDMEAN}) \tag{5}$$

where logit $P$ is the linear portion of the regression equation which is used to create the predictor model and BPDMEAN is the mean of the ratios for the "patient". The probability equation is given by:

$$P = \frac{e^{997.042-(12223.223^{*}\text{BPDMEAN})}}{1+e^{997.042-(1223.223^{*}\text{BPDMEAN})}}. \tag{6}$$

This analysis was performed using the SigmaStat (Systat Software, Inc. Richmond, CA) statistical software package.

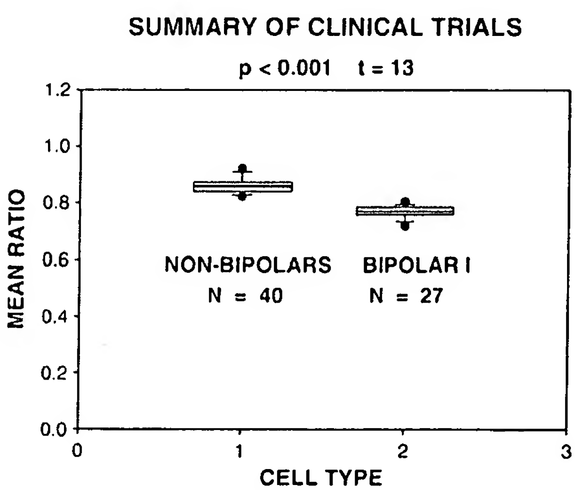


Fig. 1. This figure shows the summary of all the data collected using whole blood samples during the clinical trials. A total of 67 patient samples were tested. There were 40 negatives (non bipolar) samples and 27 positives (bipolar I) samples. Statistical analysis using the Student's $t$-test shows that the Bipolar I group is significantly different from the non-bipolar group with a $p<0.001$ and $t=13$. The data are presented in box plots. The bottom line of the rectangular box represents the 25 percentile of the data population while the top line of the box represents the 75 percentile. The lines inside the box represent the median and the mean values. Similarly the bottom cross line represents the 5 percentile and the top cross line represents the 95 percentile.

### 2.5. Box plots

The data are presented in box plots. The bottom line of the rectangular box represents the 25 percentile of the data population while the top line of the box represents the 75 percentile. The lines inside the box represent the median and the mean values. Similarly the bottom cross line represents the 5 percentile and the top cross line represents the 95 percentile (for example see Fig. 1).

## 3. Results and discussion

### 3.1. Experiments with lymphoblasts

Experimental results suggested that the fluorescence intensity of the membrane potential dye in lymphoblasts from bipolar patients was significantly different from that in lymphoblasts from siblings and normal controls. We also measured the ratio of fluorescence intensity of the membrane potential dye in the $K^+$-free buffer to that of the $K^+$-containing (reference) buffer. The ratio in unaffected cells was significantly different from that of affected cells. This result formed the basis for using the $K^+$-containing buffer as the reference buffer and the $K^+$-free buffer as the comparative buffer. To augment the difference in ratio between bipolar and non-bipolar cells we incubated these cells in ethacrynate that affects the

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ionic gradients (Rapeport et al., 1986). The fluorescent intensity ratio (fluorescent intensity with ethacrynate/fluorescent intensity without ethacrynate) was measured in reference buffer and in $K^+$-free buffer. The relative intensity ratio was determined by dividing the fluorescent intensity ratio in the $K^+$-free buffer by the ratio of intensities in the reference buffer. Both the buffers contained ethacrynate in these experiments. The relative intensity ratio of the affected cells was significantly higher than that of the unaffected cells by the $t$-test.

Discussion: Ethacrynate (a diuretic), is shown to increase the intracellular sodium and decreases the intracellular potassium (Rapeport et al., 1986). The increase in the intracellular sodium is known to activate the sodium pump activity to pump the extra sodium out of the cell. Thus, the addition of ethacrynate improved the differences between the membrane potential ratio in the blood cells from the affected patients and that in the unaffected patients. Li and El-Mallakh found that the ethacrynate has a differential effect on the $Na^+$, $K^+$-ATPase regulation in the lymphoblasts from the bipolar patients compared to the cells from the psychiatrically normal controls (Li and el Mallakh, 2004). (The supporting experimental data discussed in this section may be obtained from the corresponding author).

### 3.2. Clinical trial results and discussion

#### 3.2.1. Blind trials

During the blind trial the person who performed the membrane potential measurements knew only the sample identification number and not the patient history or the diagnosis. The mean ratio is calculated by performing the tests a minimum of six to twelve times and taking an average of the data points.

Fig. 1 shows a summary of the blind test results. The $t$-test indicates a significant difference among these two groups ($p<0.001$, $t=13$). Descriptive statistics show that the median value of the mean ratios for the non-bipolar population is 0.858 whereas that for the bipolar I population is 0.771 and that the mean value for the non-bipolar population is 0.862 while that for the bipolar I population was 0.770. These values are very close to the median values for each of these groups. The coefficient of variation ranged from 3.5 to 4.5 in all these tests. A logistic regression analysis was performed with bipolar diagnosis as outcome and the mean membrane potential ratios as the predictor. The predictor model was statistically reliable with a likelihood ratio statistic of 56.227.

Seventeen of the bipolar patients were on lithium while 9 were on valproate and one was on topiramate after electroconvulsive therapy. Two of the bipolar patients were diabetics. No significant effect of these medications on these limited tests was observed. We interpret these medication results to suggest that under the conditions used in the measurement of membrane potential (30 μl blood in 9 ml buffer; 300-fold dilution), the effect of medications including lithium on the membrane potential appears to be minimal. We have collected additional test results using a plate reader for 20 patients on lithium during the test and 17 un-medicated patients confirming these results (unpublished data on file). A comparison of the specificity and sensitivity of the results from this trial for the bipolar I group with those for schizophrenia and unipolar groups is discussed below.

#### 3.2.2. Specificity and sensitivity

Results: Specificity is the ability to identify those who do not have the disease (Dawson and Trapp, 2004). Out of a control population (those with no known mental illness) of 18, all of them were diagnosed as negative. The specificity in identifying controls was 100% as shown in Table 2. The specificity for the overall non-bipolar population (including controls, schizophrenics and unipolars) was 87.5% (35 out of 40) as shown in Table 2. Among the schizophrenic patients, the test diagnosed 8 out of 10 correctly indicating a specificity of 80%. The specificity for the unipolar patients was 75% (9 out of 12).

Table 2
Sensitivity and specificity for the clinical trial

| Summary of blind tests for bipolar disorder I | |
|---|---|
| Total samples tested: | 67 |
| Total positives: | 27 |
| Total negatives: | 40 |
| **Diagnostic blind test results** | |
| Total negatives | 40 |
| True negatives | 35 |
| Specificity | 87.5% |
| Total controls | 18 |
| True controls | 18 |
| Specificity | 100% |
| Total schizophrenics | 10 |
| True schizophrenics | 8 |
| Specificity | 80% |
| Total unipolars | 12 |
| True unipolars | 9 |
| Specificity | 75% |
| Total bipolar I | 27 |
| True bipolar I | 20 |
| Sensitivity* | 74% |

*Note: Absence of a gold standard for diagnosis of bipolar disorder I makes the sensitivity estimate unclear.

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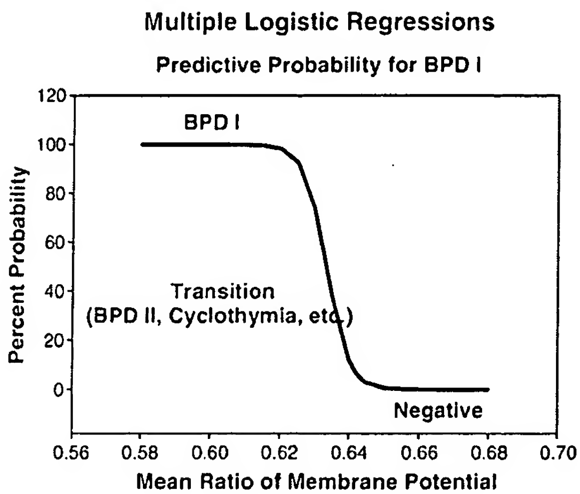
Sensitivity is defined as the test's ability to detect the illness among the patients who actually have the disease (Dawson and Trapp, 2004). Among the population of 27 bipolar I patients, 21 were true positives as per the blood test. Hence the sensitivity of the test was 78%. However the absence of a gold standard for bipolar disorder I makes the estimate of sensitivity unclear.

Discussion: A comparison of the diagnostic blood test with 16 other tests currently being used to diagnose various other diseases indicates (Cetin et al., 2001; Wallach, 2000; Okegawa et al., 2000; Kertai et al., 2003; Shlipak et al., 1999; Liu et al., 2003; Yu et al., 2003; Marchena et al., 2003; Matsuda et al., 1991; Joo et al., 2003; Vrublevskii et al., 2003; Mathiesen et al., 1998; Zeng et al., 2003; Hirose et al., 1999; Montine et al., 2001; Montgomery et al., 2000) that some tests have high specificity with low sensitivity whereas others have high sensitivity with low specificity. For example, the diagnostic test for osteoporosis (Cetin et al., 2001) has a sensitivity of only 21% whereas it has a specificity of 95% as compared to the test for systemic lupus erythematosus using antinuclear antigen which has a high sensitivity of 95% with a low specificity of only 50%. In the latter test, low specificity makes it somewhat less useful (Wallach, 2000). Caution is appropriate in comparing the bipolar I test with other well established tests using large number of subjects.




| Mean Ratio | Percent Probability |
|---|---|
| 0.67 | 0.00 |
| 0.665 | 0.01 |
| 0.66 | 0.03 |
| 0.655 | 0.14 |
| 0.65 | 0.64 |
| 0.645 | 2.86 |
| 0.644 | 3.84 |
| 0.643 | 5.13 |
| 0.642 | 6.83 |
| 0.64 | 11.86 |
| 0.635 | 38.04 |
| 0.63 | 73.69 |
| 0.625 | 92.74 |
| 0.62 | 98.31 |
| 0.615 | 99.63 |
| 0.61 | 99.92 |
| 0.605 | 99.98 |
| 0.6 | 100.00 |
| 0.59 | 100.00 |
| 0.58 | 100.00 |

Fig. 2. The binomial logistic regression model can be used to calculate the probability that the "patient" is bipolar I or not. The probability is calculated using the Eq. (6) of the text as a function of the mean ratio of membrane potentials. This relationship is shown in this figure as well as in the accompanying table for convenient use by psychiatrists.

## 4. Possible use of membrane potentials in diagnostics

The following procedure may be used to diagnose an individual patient. The patient's blood cell sample is tested as described. Six to twelve values of fluorescence intensities in $K^+$-free buffer containing ethacrynate are compared to the same number of fluorescence intensities in $K^+$-containing buffer without ethacrynate. The mean value of these ratios may be used to diagnose this particular patient by using predictive probability Eq. (6) derived from the logistic regression of a non-bipolar group and a bipolar I group. A plot of these probabilities as a function of BPDMEAN mean ratios is shown in Fig. 2 along with a table for these values. The probability, $P$, is 1 for a patient whose BPDMEAN ratio is 0.77 meaning that the probability the patient has bipolar disorder I is 100% according to this test. Thus this patient is diagnosed to be bipolar I by this test.

## 5. Further clinical trials needed

The clinical trial discussed in this paper is an essential first step toward further expanded clinical trials that are needed to cover the entire spectrum of factors including:

1. Children and adolescents,
2. Effect of medications,
3. Post-diagnostic evaluation of false positives and false negatives,
4. Larger sample size,
5. Balanced mix of patient demographics,
6. Participation of more psychiatrists in the evaluation process,
7. Inclusion of BPD II and ADHD,
8. Evaluation of trait and state influences and
9. Optimization of test parameters.

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## 6. Conclusions

The above studies show that the membrane potentials provide a useful diagnostic marker for bipolar disorder I. This could be demonstrated by regulating the potassium and sodium gradients through chemical means. The clinical trials indicate that the measurement of membrane potentials of whole blood samples could be used for differentiating the bipolar I patients from the non-bipolar patients. The specificity of the test was 87.5% and the sensitivity was 78%. These values compare well with several other currently used diagnostic tests of several diseases. Further clinical trials are needed to improve the sensitivity and reliability of this test taking into consideration the multitude of factors affecting the bipolar disorder I. Additional research is also needed to understand the mechanisms of membrane potential regulation in cells from bipolar patients which may lead o an understanding of the pathogenesis of this illness.

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Rif S. El-Mallakh, M.D., Director of Mood Disorders Program, School of Medicine, at University of Louisville, KY reviewed this manuscript and suggested several improvements. Oliver W. Hill, Jr., Ph.D. Professor and Chair, Department of Psychology, Virginia State University, Petersburg, VA suggested and assisted with the multiple regression technique used in this paper. Rama Thiruvengadam, M.D. a clinical psychiatrist in Kona, HI suggested many useful editorial changes. Douglas Woodruff, M.D. (who was the Psychiatric Editor of Stedman's Medical Dictionary, the 27th edition, and is acting in the same capacity for the present edition in progress) has also edited the revised manuscript. A patent based on the findings of this paper is pending with US patent office.

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